

SPECIFICATION

MARKER GENE FOR ARTHRORHEUMATISM TEST

Technical Field

The present invention relates to rheumatoid arthritis susceptibility genes identified de novo by a gene mapping method using microsatellite polymorphic markers, and to use thereof.

Background Art

Arthrorheumatism (Rheumatoid arthritis: RA) is a chronic inflammatory disease characterized by autoimmunity. RA, which exhibits progressive inflammation with meningeal cell overproliferation in joints, is pathologically classified into joint tissue diseases. The morbidity of RA with respect to population is high and reaches approximately 1% of various races. The familial aggregation and monozygotic twin concordance rates of RA have previously been reported to be relatively high, suggesting the presence of an inheriting factor in its pathogenesis. Indeed, it is known that in the family of a proband with RA, a closer relative of the proband has higher risk of recurrence. According to previous reports, the ratio of risk of the disease in the siblings (λ_s) of the proband falls within 2 to 10.

Among RA susceptibility genes previously found, the HLA-DRB1 locus in the HLA class III region on 6p21.3 has been thought to most strongly contribute to RA and estimated to account for 30 to 50% of total genetic risk. On the contrary, this also suggests the presence of other genes undiscovered having genetic contribution as strong as HLA-DRB1. Some of such other genes have been considered to reside in the HLA region and have linkage with HLA-DRB1. Many researchers have continuously conducted studies to identify those other genes by various approaches including genomewide linkage analysis such as sib-pair analysis (Non-Patent Documents 1 to 3) and genetic association analysis such as case-control analysis (case-control study) on candidate genes or chromosome regions (Non-Patent Documents 4 to 6). However, these studies fell short of the identification of all RA susceptibility genes and the full explanation of mechanisms of its onset.

An approach that examines the association between bases exhibiting single nucleotide polymorphisms (SNPs) in the human genomic DNA sequence and disease has received attention as a method for identifying novel disease-related genes or the like. However, SNPs are derived from one-nucleotide substitution on the genome and therefore result in only two alleles in general. In this approach, since only some SNPs, which are present within approximately 5 kb from a disease-related gene to be mapped, exhibit association, genome mapping with SNPs as polymorphic markers requires assigning an enormous number of SNPs as markers for analysis. Under

the present circumstances, this approach is therefore applied only to a limited region narrowed down to some extent. On the other hand, a microsatellite polymorphic marker has many alleles and is characterized in that it exhibits association even at some position distant from a gene to be mapped. However, the microsatellite polymorphic marker presented problems in that too many polymorphic markers assigned make analysis difficult in light of time and labors, as with SNPs, while too few polymorphic markers assigned make marker spacings too large and might overlook a disease-related gene.

The present inventors have developed a gene mapping method using microsatellite polymorphic markers assigned at approximately 50-kb to 150-kb intervals on average and have found that a region where a disease-related gene or gene relating to human phenotypes with genetic factors is present can be identified at high efficiency and low cost by using the method (Patent Document 1).

Non-Patent Document 1: Conelis, F. et al., Proc. Natl. Acad. Sci. USA, 95, 10746 (1998)

Non-Patent Document 2: Shiozawa, S. et al., Int. Immunol., 10, 1891 (1998)

Non-Patent Document 3: Jawaheer, D. et al., Am. J. Hum. Genet., 68, 927 (2001)

Non-Patent Document 4: Okamoto, K., et al., Am. J. Hum. Genet., 72, 303 (2003)

Non-Patent Document 5: Suzuki, A. et al., Nat. Genet., 34, 395 (2003)

Non-Patent Document 6: Tokuhiko, S. et al., Nat. Genet.,
35, 341 (2003)

Patent Document 1: International Publication of
WO01/79482

Disclosure of the Invention

Accordingly, an object of the present invention is to identify novel RA susceptibility genes by applying a precise mapping method with microsatellite markers capable of completely identifying disease susceptibility genes at higher cost efficiency than that of conventional approaches of SNP association analysis to multifactorial disorder RA for the first time. A further object of the present invention is to eventually develop the effective prevention/treatment of RA by collecting data on RA pathogenesis or onset mechanisms on the basis of the information of the identified RA susceptibility genes or RA-related proteins as expression products of the genes and performing proper screening.

In the present invention, a gene mapping method using microsatellite (hereinafter, referred to as "MS") was used to identify novel RA susceptibility genes whose associations with RA had not been known so far.

The RA susceptibility genes identified de novo by the present invention are TNXB and NOTCH4 genes (chromosome 6) as well as RAB6A, MPRL48, FLJ11848, UCP2, and UCP3 genes (chromosome 11) in the human genomic DNA sequence. The present inventors conducted the association analysis of RA with SNPs

present in the genomic DNA sequences of these de novo-identified genes, and found statistically significant association for the first time.

Thus, in the first aspect, the present invention provides a marker gene for arthrorheumatism test consisting of a consecutive partial DNA sequence comprising at least one base exhibiting single nucleotide polymorphism present in a TNXB, NOTCH4, RAB6A, MPRL48, UCP2 or UCP3 gene in the human genomic DNA sequence, or of a complementary strand of the partial DNA sequence.

In the second aspect, the present invention provides a test method and test kit for RA using the marker gene.

Brief Description of the Drawings

Figure 1 is a diagram showing the positions where MS markers used in Example of the present application are mapped on chromosomes;

Figure 2 is a diagram showing the mapping and P-values of MS markers used in a first-phase screening. The P-values of 133 MS markers exhibiting significance are indicated by circles (o);

Figure 3 is a diagram showing the positions where MS and SNP markers selected in Example of the present application are mapped on chromosomes, blocks predicted by EM and Clark algorithms, and P-values for allele frequency;

Figure 4 is a diagram showing the distribution of tissue expression of RA susceptibility genes identified by the present invention, and so on;

Figure 5-1 is a list showing information on the designations (left in each column) and Genbank registration numbers (right in each column) of microsatellite markers and primers used in the present invention;

Figure 5-2 is a list showing information on the designations (left in each column) and Genbank registration numbers (right in each column) of microsatellite markers and primers used in the present invention;

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Figure 5-42 is a list showing information on the designations (left in each column) and Genbank registration numbers (right in each column) of microsatellite markers and primers used in the present invention.

Best Mode for Carrying Out the Invention

A gene mapping method used in the present invention is a method described in the Patent Document 1. Specifically, this method comprises: using forward and reverse primers corresponding to each DNA sequence of consecutive DNA sequences comprising MS polymorphic markers assigned at given intervals, preferably approximately 100-kb intervals, on the human genome to amplify the DNA sequence samples by polymerase chain reaction PCR; performing electrophoresis on a high resolution gel such as a DNA sequencer; and measuring and analyzing the microsatellite polymorphic marker-containing DNA sequence fragments, which are amplification products.

MS polymorphic markers exhibiting false positive can be decreased drastically without forced correction by adopting multi-phased screening that involves performing a first (first-phase) screening using forward and reverse primers corresponding to MS polymorphic markers assigned genomewide and performing a second (second-phase) screening on MS polymorphic markers exhibiting positive in the first screening by use of a different sample population.

The position of a target gene is restricted by the multi-phased screening using MS. Then, candidate regions or gene loci can further be determined in detail by another gene mapping method. For example, analysis using SNP is effective for this purpose. Specifically, the polymorphism frequencies of SNPs in the candidate regions that appear to have the target gene are compared, for example by association analysis, between populations of patients and normal individuals, and SNP markers with linkage disequilibrium detected by haplotype analysis can be detected by linkage disequilibrium analysis.

To identify RA susceptibility genes, the present invention adopted a previously reported pooled DNA method as a screening method with good cost efficiency using 27,158 MS markers including 20,755 newly established loci. The genome association analysis was conducted by a three-phased screening method involving three major steps as described above: (1) three-phased genomic screening for reducing a type I error rate; (2) the confirmation of association of pools by individual genotyping on positive MS loci; and (3)

identification by detailed individual genotyping on SNP markers in the neighborhoods of candidate regions in screened and additional populations.

The association analysis of the whole genome demonstrated the strongest association of the HLA-DRB1 gene, which has previously been known to have association with RA ($P=9.7 \times 10^{-20}$). Furthermore, strong association was observed, independently of HLA-DRB1, in NOTCH4 ($P=1.1 \times 10^{-11}$) and TNXB ($P=7.6 \times 10^{-7}$) genes on chromosome 6 also carrying HLA-DRB1. Moreover, novel association was found in a mitochondrial-related gene cluster on 11q13.4 containing mitochondrial ribosomal protein L48 (MRPL48) and two mitochondrial proteins called uncoupling proteins (UCP2 and UCP3). Weak association was seen on 10p13 and 14q23.1. In addition to these novel associations, association was confirmed in IkBL (Non-Patent Document 4) and PADI4 (Non-Patent Document 5) genes, which have already been reported to have association with RA, as with HLA-DRB1.

Namely, statistically significant difference in allele frequencies of SNPs present in TNXB, NOTCH4, RAB6A, MRPL48, UCP2, and UCP3 genes found de novo to have association was observed between RA patients and normal individuals. Thus, a consecutive partial DNA sequence comprising at least one base exhibiting signal nucleotide polymorphism present in any of these gene regions or a complementary strand of the partial DNA sequence can be utilized as a marker gene for arthrorheumatism test.

Specifically, it is preferred that the base exhibiting single nucleotide polymorphism should be selected from the group consisting of:

the 61st base in SEQ ID NO: 1 or a corresponding base on a complementary strand thereof;

the 61st base in SEQ ID NO: 2 or a corresponding base on a complementary strand thereof;

the 61st base in SEQ ID NO: 3 or a corresponding base on a complementary strand thereof;

the 61st base in SEQ ID NO: 4 or a corresponding base on a complementary strand thereof;

the 401st base in SEQ ID NO: 5 or a corresponding base on a complementary strand thereof;

the 495th base in SEQ ID NO: 6 or a corresponding base on a complementary strand thereof;

the 61st base in SEQ ID NO: 7 or a corresponding base on a complementary strand thereof;

the 61st base in SEQ ID NO: 8 or a corresponding base on a complementary strand thereof;

the 61st base in SEQ ID NO: 9 or a corresponding base on a complementary strand thereof;

the 61st base in SEQ ID NO: 10 or a corresponding base on a complementary strand thereof;

the 401st base in SEQ ID NO: 11 or a corresponding base on a complementary strand thereof;

the 401st base in SEQ ID NO: 12 or a corresponding base on a complementary strand thereof;

the 401st base in SEQ ID NO: 13 or a corresponding base on a complementary strand thereof;

the 503rd base in SEQ ID NO: 14 or a corresponding base on a complementary strand thereof;

the 201st base in SEQ ID NO: 15 or a corresponding base on a complementary strand thereof;

the 511th base in SEQ ID NO: 16 or a corresponding base on a complementary strand thereof;

the 201st base in SEQ ID NO: 17 or a corresponding base on a complementary strand thereof;

the 51st base in SEQ ID NO: 18 or a corresponding base on a complementary strand thereof;

the 61st base in SEQ ID NO: 19 or a corresponding base on a complementary strand thereof;

the 497th base in SEQ ID NO: 20 or a corresponding base on a complementary strand thereof;

the 201st base in SEQ ID NO: 21 or a corresponding base on a complementary strand thereof; and

the 201st base in SEQ ID NO: 22 or a corresponding base on a complementary strand thereof.

SEQ ID NOs: 1 to 5 represent partial sequences of the TNXB gene, SEQ ID NOs: 6 to 13 represent partial sequences of the NOTCH4 gene, SEQ ID NO: 14 represents a partial sequence of the RAB6A gene, SEQ ID NOs: 15 to 18 represent partial sequences of the MPRL48 gene, SEQ ID NOs: 19 and 20 represent partial sequences of the FLJ11848 gene, SEQ ID NO: 21 represents

a partial sequence of the UCP2 gene, and SEQ ID NO: 22 represents a partial sequence of UCP3.

These marker genes can be used in genetic testing on RA.

For example, the consecutive DNA sequence comprising the base exhibiting single nucleotide polymorphism is amplified, for example by PCR, using forward and reverse primers positioned to keep the base exhibiting single nucleotide polymorphism in between them. Nucleotide sequences of the obtained DNA fragments can be determined and compared with a determined corresponding nucleotide sequence from a normal individual to thereby test the presence or absence of a genetic factor for RA.

The forward primer used in the test is a primer having the same nucleotide sequence as a sequence extending in the 3'-end direction from the 5' end of the DNA sequence of the marker gene containing the base exhibiting single nucleotide polymorphism, which has been mapped on the human genome, and includes those of 15 to 100 bases, preferably 15 to 25 bases, more preferably 18 to 22 bases, in length. The reverse primer is a primer having a nucleotide sequence complementary to a sequence extending in the 5'-end direction from the 3' end of the DNA sequence of the marker gene, and those of 15 to 100 bases, preferably 15 to 25 bases, more preferably 18 to 22 bases, in length can be used as the reverse primer.

Examples of primers for amplifying the marker genes having the DNA sequences of SEQ ID NOs: 1 to 22 include those having

DNA sequences represented by SEQ ID NOs: 23 to 66. The relationship of their correspondence is as follows:

Marker gene	Forward primer	Reverse primer
SEQ ID NO: 1	SEQ ID NO: 23	SEQ ID NO: 24
SEQ ID NO: 2	SEQ ID NO: 25	SEQ ID NO: 26
SEQ ID NO: 3	SEQ ID NO: 27	SEQ ID NO: 28
SEQ ID NO: 4	SEQ ID NO: 29	SEQ ID NO: 30
SEQ ID NO: 5	SEQ ID NO: 31	SEQ ID NO: 32
SEQ ID NO: 6	SEQ ID NO: 33	SEQ ID NO: 34
SEQ ID NO: 7	SEQ ID NO: 35	SEQ ID NO: 36
SEQ ID NO: 8	SEQ ID NO: 37	SEQ ID NO: 38
SEQ ID NO: 9	SEQ ID NO: 39	SEQ ID NO: 40
SEQ ID NO: 10	SEQ ID NO: 41	SEQ ID NO: 42
SEQ ID NO: 11	SEQ ID NO: 43	SEQ ID NO: 44
SEQ ID NO: 12	SEQ ID NO: 45	SEQ ID NO: 46
SEQ ID NO: 13	SEQ ID NO: 47	SEQ ID NO: 48
SEQ ID NO: 14	SEQ ID NO: 49	SEQ ID NO: 50
SEQ ID NO: 15	SEQ ID NO: 51	SEQ ID NO: 52
SEQ ID NO: 16	SEQ ID NO: 53	SEQ ID NO: 54
SEQ ID NO: 17	SEQ ID NO: 55	SEQ ID NO: 56
SEQ ID NO: 18	SEQ ID NO: 57	SEQ ID NO: 58
SEQ ID NO: 19	SEQ ID NO: 59	SEQ ID NO: 60
SEQ ID NO: 20	SEQ ID NO: 61	SEQ ID NO: 62
SEQ ID NO: 21	SEQ ID NO: 63	SEQ ID NO: 64
SEQ ID NO: 22	SEQ ID NO: 65	SEQ ID NO: 66

Alternatively, the presence or absence of an inheriting factor for RA can also be examined by using the marker genes

of the present invention as probes to screen a DNA sample from a test subject, determining a nucleotide sequence of the obtained DNA of the test subject, and then comparing the sequence with a sequence from a normal individual.

In this context, the probe used may be the marker gene of the present invention itself or may be the consecutive DNA sequence comprising the base exhibiting single nucleotide polymorphism present in the marker gene, a complementary strand thereof, or sequences hybridized by them. Preferably, a probe of 15 to 100 bases, preferably 15 to 25 bases, more preferably 18 to 22 bases, in length can be used.

On the other hand, coding regions encoded by the RA susceptibility genes can be determined by determining the full-length nucleotide sequences of the RA susceptibility genes on the basis of TNXB, NOTCH4, RAB6A, MPRL48, UCP2 and UCP3 genes found de novo to have association. As a result, amino acid sequences of proteins encoded by the genes can be identified. Since proteins with these amino acid sequences are highly likely to participate in RA pathogenesis or onset mechanisms, RA can be prevented or treated by promoting or inhibiting the functions of these proteins.

Thus, the present invention also relates to a screening method using the proteins. Substances promoting or isolating the functions of the proteins, that is, agonists or antagonists can be identified by the screening method. The antagonist used herein encompasses not only chemical small molecules but also biologically relevant substances such as antibodies,

antibody fragments, and antisense oligonucleotides. These agonists or antagonists are effective as diagnostic, preventive, and/or therapeutic drugs for RA.

The protein described above may be produced in a transformed cell obtained by preparing a vector comprising a DNA sequence containing at least the coding region of any of the marker genes identified by the present invention, and then transforming the vector into an appropriate host cell.

Example

Microsatellite (MS) Detection and PCR Primer Design:

MS sequences with 2-, 3-, 4-, 5-, or 6-base repeat units were detected with Apollo program applicable to Sputnik in four versions of the human genome draft sequences from Golden Path Oct. 2000 to NCBI build 30. PCR primers for amplifying these repeats under single reaction conditions were automatically designed with Discover program applicable to PrimerExpress. To prevent differential amplification, these PCR primers were designed to contain no SNP in their sequences (Sham et al, 2002).

A pattern with a number of peaks exhibiting the polymorphisms of MS markers in a pool of Japanese (Barcellos. L. F. et al., Am. J. Hum. Genet., 61, 734 (1997)) was compared with that of European pools. As a result, individual polymorphic MS markers in the Japanese pool exhibited a different pattern from that of two European pools (data not shown). The result of the comparison between the races showed

that the pattern with a number of peaks in the Japanese pool reflects polymorphism in MS length and is not experimental error.

In the present invention, 27,158 polymorphic MS markers were assigned and mapped on the human draft sequence (NCBI build 30) (Figure 1). Among these markers, 20,755 markers were assigned de novo by the present inventors, while remaining 6,403 were known markers such as Genethon and CHLC markers. The average heterozygosity and average allele number of 27,039 markers except for 119 markers mapped on the Y gene were 0.67 ± 0.16 and 6.4 ± 3.1 , respectively. The average marker spacing thereof was 108.1 kb (SD=64.5 kb; max=930.1 kb) (see Table 1). These markers can detect linkage disequilibrium up to approximately 50 kb distant from a disease locus at a rough estimate. Accordingly, these markers were used to conduct case-control association analysis on RA. Those 27,039 microsatellites and primer sequences used for their amplification were deposited in Genbank as registration numbers listed in Figure 5.

[Table 1]

Supplementary Table 1. Microsatellite marker spacing

Chromosome	Spacing (kb)		
	Average	SD	Max.
1	104.7	64.5	581.7
2	103.6	61.2	521.4
3	103.0	67.0	766.7
4	113.0	68.4	522.4
5	107.2	61.9	461.8
6	108.6	58.3	428.5
7	100.2	62.4	634.6
8	108.3	68.6	587.3
9	106.4	65.5	930.1
10	106.6	62.6	510.2
11	104.8	64.6	463.0
12	106.8	66.6	674.2
13	108.3	57.4	356.6
14	115.9	52.6	350.2
15	119.1	67.4	625.9
16	114.1	77.3	526.0
17	112.1	70.9	546.4
18	106.5	63.6	563.7
19	110.8	69.9	421.8
20	108.6	56.9	337.0
21	108.2	58.0	378.7
22	120.3	68.1	505.7
X	123.5	65.5	443.5
Total	108.1	64.5	930.1

Microsatellite markers were mapped on the NCBI build 30.

In the present invention, 940 test subjects with RA (case population) and the same number of normal test subjects (control population) were adopted. By permission of the ethical committee of each organization associated with the present invention, informed consent was obtained from each test subject in the case and control populations used in this analysis. RA phenotypes were determined according to American Rheumatism Association diagnostic criteria for RA. All personal data associated with medical information and blood

samples were carefully discarded in organization which collected them.

Average age at disease onset in the case population was 47.7 ± 13.1 years old, with the sex ratio of 1:4 (male:female). The average age and sex ratio of the case and control populations were set as equally as possible. The sexes of all samples involved were confirmed by amelogenin (enamel protein) genotyping (Akane, A., et al., *Forensic Sci. Int.*, 49, 81 (1991)). Preliminary PCR test for checking DNA levels was conducted by PCR direct sequencing as previously reported (Voorter, C. E. et al., *Tissue Antigens*, 49, 471 (1997)), while HLA-DRB1 genotypes were examined.

DNA Sample Preparation and Typing:

DNA was extracted with QIAamp DNA blood kit (QIAGEN) from the sample of each test subject in the populations under standardized conditions for preventing variations in DNA level. Subsequently, to check DNA degradation and RNA contamination, 0.8% agarose gel electrophoresis was performed. After optical density measurement for checking protein contamination, the DNA concentration was determined by three measurements using PicoGreen fluorescence assay (Molecular Probes) as previously described (Collins, H. E. et al., *Hum. Genet.*, 106, 218 (2000)). The standardized pipetting and dispensation of the DNA samples were performed with robots such as Biomek 2000 and Multimek 96 (Beckman).

The pooled DNA template for typing two groups of approximately 30,000 MS markers was prepared simultaneously with or immediately after the DNA quantification. The pooled DNA level was further tested by comparing allelic distribution between individuals and pooled typing results using three MS markers. After this test, approximately 30,000 PCR reaction mixtures containing all the components except for the PCR primers were prepared and subsequently dispensed to 96-well PCR reaction plates, followed by storage until use.

After PCR reaction, pooled MS typing and individual genotyping were conducted according to standard protocols using ABI3700 DNA analyzer (Applied Biosystems). The pooled DNA typing could maintain constant accuracy throughout the experiment by using the standardized preparation method. Various data such as peak positions and heights were automatically read by the PickPeak and MultiPeaks programs developed by Applied Biosystems Japan, from the multipeak pattern in the chromatograph files, that is, ABI fsa files.

Three-Phased Genome Screening by Pooled DNA Method:

A population of 375 individuals with RA (case) and the same number of unaffected individuals (control) were equally divided into three pairs of case and control populations (125 individuals each). Population stratification test was conducted using 22 randomly selected microsatellites sufficient at least for population stratification according to Pritchard's method (Pritchard, J. K. and Rosenberg, N. A.,

Am. J. Hum. Genet., 65, 220 (1999)). The results showed the absence of any significant stratification in either case or control populations (Table 2). The prevention of false association by the population stratification test is very important for late-onset diseases such as RA (Risch 2000) where the collection of internal controls is difficult.

[Table 2]

Supplementary Table 2. Stratification test among case and control populations

Chr.	Markers	Fisher's exact P values														
		1st screening (n=125)				2nd screening (n=125)				3rd screening (n=125)				Additional samples (n=565)		Total (n= 940)
		2x2	# of Allele	2cm		2x2	# of Allele	2cm		2x2	# of Allele	2cm		2x2	# of Allele	
1	D1S0368I	0.296	6	0.780	0.113	5	0.181	0.248	7	0.524	0.331	8	0.939	0.093	9	0.827
2	D2S1338	0.171	8	0.555	0.070	8	0.034	0.059	9	0.480	0.250	10	0.585	0.453	10	0.860
3	D3S2439	0.025	9	0.351	0.015	10	0.073	0.040	9	0.414	0.028	11	0.503	0.184	11	0.775
4	G10243	0.387	7	0.884	0.268	7	0.906	0.095	7	0.233	0.078	8	0.202	0.020	8	0.063
5	D5S0029I	0.857	3	0.960	0.710	3	0.933	0.068	5	0.172	0.186	4	0.399	0.146	5	0.415
6	G10114	0.373	8	0.852	0.038	6	0.272	0.282	7	0.464	0.012	6	0.036	0.255	8	0.468
7	D7S1802	0.157	8	0.446	0.157	9	0.664	0.258	8	0.882	0.009	11	0.230	0.013	12	0.232
8	HUMUT1239	0.022	7	0.034	0.399	7	0.814	0.123	7	0.466	0.098	6	0.453	0.282	8	0.815
9	D8S0147I	0.372	7	0.717	0.125	6	0.341	0.238	7	0.279	0.158	7	0.733	0.168	8	0.513
10	G08808	0.210	8	0.541	0.074	7	0.123	0.123	6	0.475	0.030	9	0.048	0.168	10	0.220
11	D11S0689I	0.215	10	0.595	0.062	10	0.124	0.553	9	0.886	0.308	13	0.902	0.148	13	0.699
12	G08964	0.248	8	0.286	0.071	7	0.109	0.229	8	0.673	0.146	9	0.920	0.333	9	0.820
13	D13S0102I	0.089	6	0.476	0.135	6	0.368	0.187	8	0.256	0.001	7	0.016	0.002	7	0.020
14	D14S608	0.015	10	0.163	0.253	8	0.800	0.340	10	0.887	0.051	10	0.164	0.028	10	0.114
15	G07912	0.194	6	0.306	0.179	6	0.602	0.248	9	0.624	0.207	7	0.827	0.076	9	0.284
16	D16S0026I	0.107	8	0.698	0.358	9	0.762	0.136	10	0.658	0.037	11	0.389	0.047	11	0.443
17	D17S0044I	0.104	16	0.888	0.361	15	0.899	0.061	12	0.415	0.064	23	0.708	0.053	24	0.590
18	D18S0013I	0.123	9	0.299	0.499	8	0.933	0.172	8	0.280	0.315	10	0.961	0.382	10	0.920
19	D19S0019I	0.498	8	0.977	0.216	7	0.564	0.078	8	0.365	0.036	8	0.181	0.290	8	0.581
20	D20S0030I	0.247	7	0.557	0.047	7	0.149	0.339	7	0.724	0.503	8	0.970	0.125	9	0.534
21	D21S0036I	0.036	13	0.558	0.109	11	0.339	0.050	11	0.099	0.060	13	0.365	0.059	15	0.628
22	D22S0155I	0.246	4	0.443	0.003	4	0.010	0.124	4	0.207	8.07E-05	4	3.44E-04	0.001	4	0.010
X	HUMUT1223	0.115	5	0.515	0.140	6	0.416	0.122	5	0.182	0.006	6	0.069	0.021	6	0.081
Pritchard's chi square		69.4	81.8	65.4	70.0	54.8										54.8
df		65	64	62	65	65										65
P value		0.331	0.068	0.361	0.314	0.818										0.818

After the population stratification test, three pooled DNA templates from each case or control population were used in three-phased genomic screening. This screening method simply means reproduction in three independent sample populations and is known to be suitable for excluding many false positives due to Type I errors caused by multiple testing (Barcellos, L. et al., Am. J. Hum. Genet., 61, 724 (1997)). The first (first-phase) screening indicated that 2,847 MS markers were statistically significant ($P < 0.05$) by the Fisher's exact test for either 2×2 or $2 \times m$ contingency tables (m =the number of alleles). Subsequent second (second-phase) screening indicated that of these 2,847 markers, 372 MS markers were significant. After further third (third-phase) screening, 133 positive MS markers were obtained. These results are shown in Table 3.

[Table 3]

Chromosome	Screening phases					
	1st (n=125 each)		2nd (n=125 each)		3rd (n=125 each)	
	Marker	Number of Positive*	Marker	Number of Positive	Marker	Number of Positive
1	2,241	232	232	27	25	10
2	2,373	249	249	29	25	7
3	1,991	204	204	33	30	8
4	1,740	184	184	23	23	11
5	1,733	168	168	22	20	11
6	1,619	170	170	36	29	10
7	1,599	201	201	25	23	9
8	1,375	124	124	14	11	4
9	1,101	135	135	9	9	4
10	1,281	127	127	18	16	7
11	1,303	139	139	16	13	5
12	1,260	144	144	16	12	3
13	893	99	99	12	9	6
14	762	79	79	19	19	4
15	689	71	71	9	7	4
16	732	57	57	12	13	5
17	725	77	77	6	4	2
18	750	86	86	8	7	5
19	503	67	67	10	11	3
20	565	50	50	7	7	2
21	324	37	37	4	4	1
22	293	33	33	6	5	4
X	1,187	114	114	11	13	8
Total	27,039	2,847 (1,377)	2,847	372 (215)	335	133 (53)

*Number of positive markers by the Fisher exact test for the 2x2 or 2xm contingency tables. The number of positive markers by 2xm is indicated in parenthesis.

The number of the positive MS markers was larger than statistically expected, suggesting that experimental errors caused by the pooled DNA method were contained therein, as previously reported (Shaw, S. H. et al., Genome Res., 8, 111 (1998); and Sham, P. et al., Nat. Rev. Genet., 3, 862 (2002)). Thus, we carefully verified these positive markers by individual genotyping in the screened populations. As a result, 47 markers were significant. Of these markers, 25

were excluded due to their low positive allele frequencies (<0.05), resulting in a list of 23 positive MS markers (Table 4).

[Table 4]

Table 1. Twenty-five positive microsatellite markers from individual genotyping

Markers	Cytobands	Number of allele	Allele frequencies		Case	Fisher's exact P values		Odds Ratio	95%CI
			Control	Positive		2x2	PC		
D6S0588i	6p21.3	10	0.430	5	0.572	0.00000055	0.000014	1.78	1.45 - 2.18
D6S0483i	6p21.3	18	0.089	7	0.176	0.00000082	0.00024	2.18	1.59 - 2.98
D6S1061	6p21.3	24	0.095	16	0.183	0.0000001	0.00026	2.14	1.57 - 2.90
D11S0497i	11q13.4	5	0.513	2	0.613	0.0000031	0.008	1.55	1.26 - 1.91
D6S0025i	6p21.3	6	0.125	2	0.185	0.002	0.51	1.59	1.20 - 2.11
D10S0168i	10p13	4	0.408	2	0.499	0.0005	0.13	1.44	1.18 - 1.77
D14S0452i	14q23.1	9	0.370	4	0.452	0.001	0.26	1.40	1.14 - 1.72
D8S0127i	8q13.3	16	0.116	3	0.069	0.002	1	0.57	0.40 - 0.81
D7S0086i	7p21.1	11	0.095	4	0.053	0.002	1	0.54	0.36 - 0.80
D10S0607i	10q26.13	5	0.827	1	0.882	0.003	1	1.59	1.19 - 2.14
D13S0561i	13q31.1	10	0.130	8	0.183	0.005	1	1.50	1.13 - 2.00
G08462	5q14.1	9	0.190	4	0.136	0.005	1	0.67	0.51 - 0.89
D16S0496i	16q12.2	10	0.204	7	0.267	0.005	1	1.41	1.11 - 1.79
D5S0228i	5q12.1	11	0.305	7	0.371	0.004	1	1.35	1.09 - 1.67
D5S400	5q34	18	0.063	2	0.101	0.008	1	1.69	1.15 - 2.46
D6S0811i	6q22.33	6	0.445	3	0.515	0.008	1	1.31	1.07 - 1.61
D20S910	20p12.1	14	0.301	7	0.365	0.009	1	1.34	1.08 - 1.66
D4S0017i	4q25	22	0.071	5	0.111	0.009	1	1.64	1.14 - 2.35
D16S0232i	16q24.1	4	0.444	2	0.380	0.01	1	0.77	0.63 - 0.95
D3S1500i	3p24.3	4	0.781	1	0.725	0.01	1	0.74	0.58 - 0.84
D20S470	20p12.1	14	0.111	7	0.073	0.02	1	0.64	0.45 - 0.91
DXS0486i	Xq25	8	0.118	1	0.090	0.09	1	0.68	0.51 - 1.04
D18S0090i	18q12.1	20	0.193	13	0.153	0.05	1	0.76	0.58 - 0.99

PC means corrected P values by Bonferroni's correction.

The Fisher's exact test was carried out in the case and control populations (n=375 each). This means allele frequency of which has the lowest P value in the locus.

Specific data serving as a basis for Table 4 are shown in Table 5. As an example, this table classifies the region determined by each MS marker as positive (+) (which was judged as having significant disease association in the rheumatoid arthritis group (P) as compared with the normal individual group (C)) or as negative (-). For example, "+/+" means that both alleles are positive, and "+/-" means that one of alleles is positive, according to the classification. The use of this table allows for, for example, the digitization of the possibility of rheumatoid arthritis onset by grading each test subject according to specific algorithm on the basis of these numeric values. In the table, "o" denotes mistyping.

[Table 5]

		D6S0588i		D6S1061		D6S0483i		D6S0025i		D11S0497i		D10S0168i		D14S0452i	
		C	P	C	P	C	P	C	P	C	P	C	P	C	P
-	-	274	192	311	254	314	255	317	290	219	158	299	287	350	311
+	+	199	316	5	11	14	18	37	54	277	340	190	202	155	174
+	-	450	421	57	110	43	101	20	31	428	435	446	450	409	448
o	o	15	52	565	606	567	607	564	606	14	48	3	42	24	48
Total		938	981	938	981	938	981	938	981	938	981	938	981	938	981

		D8S0127i		D7S0086i		D10S0607i		D13S0561		G08462		D16S0496i		D5S0228i		D5S400	
		C	P	C	P	C	P	C	P	C	P	C	P	C	P	C	P
-	-	727	783	793	798	20	16	650	647	617	662	568	514	189	152	789	754
+	+	17	12	8	5	672	692	42	54	40	18	61	61	43	55	8	7
+	-	163	155	125	117	238	227	215	220	272	245	292	355	142	168	130	175
o	o	31	9	12	19	8	4	31	18	9	14	17	9	564	564	11	3
Total		938	939	938	939	938	939	938	939	938	939	938	939	938	939	938	939

		D6S0811i		D20S910		D4S0017i		D16S0232		D3S1500i		D20S470		D18S0090i		DXS0486i	
		C	P	C	P	C	P	C	P	C	P	C	P	C	P	C	P
-	-	270	250	215	194	324	301	286	352	68	64	296	324	244	268	296	318
+	+	209	241	65	93	3	9	170	152	528	471	5	4	13	8	13	7
+	-	449	437	93	88	47	65	479	430	318	351	73	47	117	99	63	50
o	o	10	11	565	564	564	564	3	5	24	53	564	564	564	564	566	564
Total		938	939	938	939	938	939	938	939	938	939	938	939	938	939	938	939

The seven most significant markers in the list of Table 4 were also significant after Bonferroni's correction

($P < 0.05$). Therefore, in this Example, SNP genotyping was focused on these candidate regions.

SNP Genotyping:

Among the seven most significant markers, four (i.e., the first, second, third, and fifth) were located in the HLA region on 6p21.3 (Figure 3), whereas the fourth, sixth, and seventh significant markers were located on 11q13.4, 10p13, and 14q23.1, respectively (cytobands are designated under the NCBI build 30).

SNPs in the neighborhoods of these candidate regions were selected from dbSNP database of NCBI homepage and JSNP database of the homepage of The Institute of Medical Science, The University of Tokyo. These SNPs were genotyped using TaqMan assay or direct sequencing. The TaqMan assay was conducted using the standard protocol of ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) equipped with 384-Well Block Module and Automation Accessory. The direct sequencing of the PCR products was conducted according to a standard approach using ABI3700 DNA analyzer (Applied Biosystems). In the HLA region, additional SNPs were selected from IkBL to C4B genes in order to verify previously reported RA association around the centromeric end of the HLA class III region. See Table 6 for the details of the selected SNPs.

Genotyping was conducted on 165 SNPs in the case and control populations used in the MS typing. Of these SNPs, 41 were neither polymorphic nor STSs (sequence tagged sites)

(see Table 6) and were therefore excluded from subsequent analysis. Among the remaining 124 SNPs, 54 were statistically significant by case-control association analysis ($P < 0.05$) (Table 7). LD block structures were predicted for these 124 SNPs by EM algorithm (Figure 2), and case-control association analysis using haplotypes in each block was conducted according to this algorithm (Table 8). To reproduce these SNP allelic associations, these 54 positive SNPs were genotyped in additional populations composed of 565 case individuals and 565 control individuals. Finally, 45 positive SNPs were obtained in the combined ($n = 2 \times 940$) population consisting of all the samples used in this experiment. Among these positive SNPs, 24 was also significant ($P_c < 0.05$) after Bonferroni's correction (Table 7).

Hereinafter, the analysis result of each chromosome will be described.

6p21.3

In the HLA region on 6p21.3, 28 of 71 polymorphic SNPs were statistically significant ($P < 0.05$) in the first test. Preliminary genotyping on HLA-DRB1 revealed that the HLA-DRB1*0405 allele was most significant ($P = 1.3 \times 10^{-12}$). The result was, as expected, consistent with many previous reports on Japanese populations (Wakitani, S. et al., Br. J. Rheumatol., 36, 630 (1997); and Shibue, T. et al, Arthritis Rheum., 43, 753 (2000)) and demonstrated that the method used in the present invention is effective for detecting the association of

susceptibility genes with RA. In addition to HLA-DRB1, the association of the IkBL gene (MIM*601022) promoter SNP rs3219185 was also reproduced ($P=5.4 \times 10^{-5}$), albeit with relatively low frequency of the minor allele. Moreover, strong association was seen around the NOTCH4 (MIM*164951) and TNXB (MIM*600985) genes, which were approximately 250 kb and 300 kb, respectively, distant from HLA-DRB1.

The NOTCH4 gene is one of proto-oncogenes with epidermal growth factor (EGF) repeats. NOTCH4 encodes a large transmembrane receptor predicted to be involved in the signal transduction of cell proliferation, cell differentiation, and angiogenesis (Yung Yu, C. et al., Immunol. Today, 21, 320 (2000)). In NOTCH4, nine SNPs were statistically significant, among which two caused amino acid exchange. Among these nine SNPs, rs2071282, the SNP in exon 4, exhibited the strongest association ($P=3.1 \times 10^{-8}$) and caused Leu203Pro exchange at the fourth EGF repeat in the extracellular domain of NOTCH4. On the other hand, rs915894 in exon 3 was moderately significant ($P=0.044$) and caused Lys116Gln exchange at the third EGF repeat.

The TNXB gene encodes one of extracellular matrix proteins with 34 fibronectin type III-like (FNIII) and 18 EGF repeats and participates in at least one of essential functions of collagen deposition in connective tissues (Mao, J. R. et al., Nat. Genet. 30, 421 (2002)). In TNXB, five SNPs were statistically significant, of which four caused amino acid exchange. Among these five SNPs, rs185819 in exon 10 exhibited

the strongest association ($P=6.8 \times 10^{-5}$) and caused His1248Arg exchange at the seventh FNIII repeat. Other SNPs, rs2075563 (Glu3260Lys) in exon 29, rs2269428 (His2363Pro) in exon 21, and rs3749960 (Phe2300Tyr) in exon 20, were also significant and located in the 26th, 18th, and 17th FNIII repeats, respectively.

These six positive SNPs were finally confirmed in the combined ($n=2 \times 940$) population (Table 6). Further, haplotype analysis demonstrated these results for IkBL, NOTCH4, and TNXB (Table 7), indicating the absence of, in all blocks of each gene, common haplotypes with greater risks than that of single SNP in each gene. When multiple logistic regression analysis was conducted for the SNPs in IkBL, TNXB, and NOTCH4 with those in HLA-DRB1, three genes, DRB1*0405 (ORs=2.29-8.84), rs3219185 in IkBL (ORs=1.16-2.67), and rs185819 in TNXB (ORs=1.00-1.62), were significant ($P<0.05$) in a partially recessive model. Two SNPs, DRB1 (ORs=2.16-4.69) and TNXB (ORs=1.02-1.84), were significant in a partially dominant model. On the other hand, when the analysis was limited to the shared epitope (SE) of DRB1, SE (ORs=1.79-3.85), IkBL (ORs=1.11-2.54), and rs2071282 in NOTCH4 (ORs=1.13-7.14) were significant only in the partially recessive model. These results suggested that these loci independently correspond to RA in the partially recessive model.

11q13.4

The candidate region on 11q13.4 contained nine genes including three mitochondrial-related genes MRPL48, UCP2, and UCP3. Although MRPL48 was recently found as a gene having homology to mammalian mitochondrial ribosomal proteins (MRPs) (Zhang, Z. and Gerstein, M., *Genomics*, 81, 468 (2003)), its function is still unknown. UCP2 (MIM*601693) and UCP3 (MIM*602044) encode transporter proteins on the inner mitochondrial membrane and participate in energy consumption. UCP2 is also known as a susceptibility gene for obesity and diabetes. RAS-associated protein RAB6A (MIM*179513) was centromerically found with respect to MRPL48. Further, three novel genes were located in regions FLJ11848, LOC374407, and DKFZP586P0123. FLJ11848 has WD40 repeats and widely participates in cell-cell interaction (Smith, T. F. et al., *Trends Biochem. Sci.*, 24, 181 (1999)). LOC374407 has been found to have homology to heat shock protein 40 homolog (HSP40 homolog) and structural similarity to spermatogenesis apoptosis-related protein. DKFZP586P0123 has one protein kinase C conserved region.

In these genes, 16 of 25 polymorphic SNPs were statistically significant in the first test. Although these positive SNPs were scattered over the region tested, most significant associations ($P=0.00015$) were observed in two SNPs, rs1792174 in 5'-UTR and rs1792160 in intron 3 of MRPL48. MRPL48 also had two other positive SNPs, rs1792193 ($P=0.003$) in intron 5 and rs1051090 ($P=0.007$) in 3'-UTR. Positive SNPs were also

observed in all of other genes UCP2, UCP3, RAB38 and FLJ11848. However, only one common haplotype in the block b2 containing MRPL48 and FLJ11848 exhibited significant association as strong as the single SNP in MRPL48. These positive SNPs in MRPL48 were finally confirmed after Bonferroni's correction in the combined population (Table 7). On the other hand, rs1527302 in DKFZP586P0123 was significant ($P=0.00078$) both in the first test and after haplotype analysis. However, the SNP allelic association was not confirmed in the combined population. These results suggested that other causative SNPs are present in the block b2.

10q13, 14q23.1, and PADI4

The candidate region on 10p13 had two genes, DKFZP761F241 and optineurin (OPTN). Three SNPs in the DKFZP761F241 gene were statistically significant in the first test and however, was not confirmed after correction in the combined population. No common haplotype existed in regions that remained after Bonferroni's correction in each population.

On the other hand, the candidate region on 14q23.1 contained only reticulon 1 gene (MIM*600865), which encodes the neuroendocrine-specific protein group. Even after Bonferroni's correction in the combined samples, rs2182138 in intron 3 of RTN1 was still statistically significant ($P=0.0002$). No common haplotype was observed in both regions that remained after correction.

Further, in the PADI4 gene that appeared to be a susceptibility gene for RA by the candidate gene approach (Non-Patent Document 5), four positive SNPs, padi89 ($P=0.002$), padi90 ($P=0.004$), rs874881 ($P=0.002$), and rs2240340 ($P=0.002$), were replicated in the populations of this Example. D1S1144i, a CA microsatellite marker in intron 6 of the PADI4 gene, was confirmed to be included in the RA marker set and exhibit slight significance ($P=0.008$) but low associated allele frequency ($P=0.037$ in the control population).

[Table 6]

Supplementary Table 4. List of all SNPs

Cyto- bands	SNPs	Method	g-SNP	Location gene name portion	Note
6p21.3	rs1615639	TaqMan(AbD)*1	cSNP	MC8 intron	
	rs2242955	TaqMan(AbD)	cSNP	BAT1 intron	
	rs2071595	TaqMan(AbD)	cSNP	BAT1 intron	
	rs3219186	Sequencing*	cSNP	BSL promoter	
	rs3219185	Sequencing	cSNP	BSL promoter	
	rs3219184	Sequencing	cSNP	BSL promoter	
	rs2071592	Sequencing	cSNP	BSL promoter	
	rs2235706	Sequencing	cSNP	BSL intron	
	rs2071591	Sequencing	cSNP	BSL intron	
	rs769178	TaqMan(AbD)*3	cSNP	AF1 intron	
11q13.4	rs2269475	TaqMan(AbD)	cSNP	AF1 intron	
	rs2657893	TaqMan(AbD)	cSNP	BAT2 syn	Not polymorphic
	rs1330771	TaqMan(AbD)	cSNP	BAT2 syn	
	rs1046069	TaqMan(AbD)	cSNP	BAT2 intron	
	rs2242956	TaqMan(AbD)	cSNP	BAT3 intron	
	rs605299	TaqMan(AbD)	cSNP	BAT3 intron	
	rs605283	TaqMan(AbD)	cSNP	OBor47 5'UTR	
	rs2142234	TaqMan(AbD)	cSNP	LYG58 intron	Not polymorphic
	rs605287	TaqMan(AbD)	cSNP	LYG58 intron	
	rs605273	TaqMan(AbD)	cSNP	BAT5 intron	
10p13	rs2242953	TaqMan(AbD)	cSNP	LYG58 intron	
	rs400547	TaqMan(AbD)	cSNP	CLIC1 intron	
	rs1150793	TaqMan(AbD)	cSNP	MSH5 intron	
	rs1707936	TaqMan(AbD)	cSNP	OBor27 intron	
	rs1707929	TaqMan(AbD)	cSNP	OBor27 intron	
	rs2242958	TaqMan(AbD)	cSNP	LSM2 intron	
	rs2075200	TaqMan(AbD)	cSNP	HSPA11 intron	
	rs2227955	TaqMan(AbD)	cSNP	HSPA11 intron	
	rs2242967	TaqMan(AbD)	cSNP	HSPA1A 5'UTR	
	rs605203	TaqMan(AbD)	cSNP	OBor46 5'UTR	Not polymorphic
11q13.4	rs2072679	TaqMan(AbD)	cSNP	C2 syn	
	rs1042863	TaqMan(AbD)	cSNP	C2 syn	Not polymorphic
	rs763303	TaqMan(AbD)	cSNP	BF syn	
	rs1046709	TaqMan(AbD)	cSNP	SRV2L intron	
	rs444821	TaqMan(AbD)	cSNP	DOM3Z intron	
	rs174534	TaqMan(AbD)	cSNP	THO3 intron	Only heterozygote
	rs2075984	TaqMan(AbD)	cSNP	THO3 syn	
	rs2242959	TaqMan(AbD)	cSNP	THO3 intron	
	rs2075983	TaqMan(AbD)	cSNP	THO3 intron	
	rs2269478	TaqMan(AbD)	cSNP	THO3 intron	
11q13.4	rs3749980	TaqMan(AbD)	cSNP	THO3 intron	
	rs3749982	TaqMan(AbD)	cSNP	THO3 syn	Only heterozygote
	rs204677	TaqMan(AbD)	cSNP	THO3 intron	
	rs105619	TaqMan(AbD)	cSNP	THO3 intron	
	rs204620	TaqMan(AbD)	cSNP	THO3 intron	
	rs204696	TaqMan(AbD)	cSNP	THO3 intron	
	rs291150	TaqMan(AbD)	cSNP	THO3 intron	
	rs204696	TaqMan(AbD)	cSNP	THO3 intron	
	rs2071289	TaqMan(AbD)	cSNP	EGFL8 intron	
	rs408356	TaqMan(AbD)	cSNP	AGPAT1 intron	
11q13.4	rs2070300	TaqMan(AbD)	cSNP	AGER intron	
	rs2071287	TaqMan(AbD)	cSNP	NOTCH4 intron	
	rs206018	TaqMan(AbD)	cSNP	NOTCH4 intron	
	rs2848012	TaqMan(AbD)	cSNP	NOTCH4 intron	
	rs422951	TaqMan(AbD)	cSNP	NOTCH4 intron	
	rs230592	Sequencing	cSNP	NOTCH4 intron	
	rs230598	Sequencing	cSNP	NOTCH4 syn	
	rs2071284	Sequencing	cSNP	NOTCH4 intron	
	rs2071283	Sequencing	cSNP	NOTCH4 syn	
	rs2071282	TaqMan(AbD)	cSNP	NOTCH4 intron	
11q13.4	rs2071281	Sequencing	cSNP	NOTCH4 syn	
	rs415929	Sequencing	cSNP	NOTCH4 syn	
	rs915894	TaqMan(AbD)	cSNP	NOTCH4 intron	
	rs431198	TaqMan(AbD)	cSNP	NOTCH4 syn	
	rs387386	Sequencing	cSNP	NOTCH4 5'UTR	
	rs1332953	TaqMan(AbD)	cSNP	OBor10 intron	Only heterozygote
	rs391123	TaqMan(AbD)	cSNP	OBor10 intron	
	rs2272019	TaqMan(AbD)	cSNP	OBor10 intron	
	rs2073044	TaqMan(AbD)	cSNP	OBor10 intron	
	rs2294578	TaqMan(AbD)	cSNP	STX2 intron	
11q13.4	rs2070523	TaqMan(AbD)	cSNP	STX2 intron	
	rs133344	TaqMan(AbD)	cSNP	Not polymorphic	
	rs120955	TaqMan(AbD)	cSNP	Not polymorphic	
	rs2227139	TaqMan(AbD)	cSNP	Not polymorphic	
	rs1054555	TaqMan(AbD)	cSNP	HLA-DQB3 intron	Multi cluster
	rs3830130	TaqMan(AbD)	cSNP	HLA-DQB3 intron	Multi cluster
	rs382815	TaqMan(AbD)	cSNP	HLA-DQB3 intron	Multi cluster
	rs3828940	TaqMan(AbD)	cSNP	HLA-DQB3 intron	Multi cluster
	rs3830121	TaqMan(AbD)	cSNP	HLA-DQB3 intron	Multi cluster
	rs2858984	TaqMan(AbD)	cSNP	HLA-DQB3 intron	Multi cluster
11q13.4	rs2238799	TaqMan(AbD)	cSNP	HLA-DQA1 intron	Multi cluster
	rs1315000	TaqMan(AbD)	cSNP	HLA-DQA1 intron	Multi cluster
	rs2847012	TaqMan(AbD)	cSNP	HLA-DQA1 intron	Multi cluster
	rs2858999	TaqMan(AbD)	cSNP	HLA-DQA1 intron	Multi cluster
	rs2071798	TaqMan(AbD)	cSNP	HLA-DQB2 intron	
	rs1049110	TaqMan(AbD)	cSNP	HLA-DQB2 intron	
	rs2071350	TaqMan(AbD)	cSNP	HLA-DQB2 intron	
	rs3781909	TaqMan(AbD)	cSNP	PLEKHB1 intron	
	rs2000734	TaqMan(AbD)	cSNP	PLEKHB1 3'UTR	
	rs6590	TaqMan(AbD)	cSNP	PLEKHB1 3'UTR	Not polymorphic
11q13.4	rs3182799	Sequencing	cSNP	RAB6A 3'UTR	
	rs3741142	Sequencing	cSNP	RAB6A 3'UTR	
	rs3182792	Sequencing	cSNP	RAB6A syn	Not polymorphic
	rs3182790	Sequencing	cSNP	RAB6A syn	Not polymorphic
	rs3182789	Sequencing	cSNP	RAB6A syn	Not polymorphic
	rs3182788	Sequencing	cSNP	RAB6A syn	Not polymorphic
	rs1464908	TaqMan(AbD)	cSNP	RAB6A intron	
	rs3203705	TaqMan(AbD)	cSNP	RAB6A intron	Only heterozygote
	rs2140993	TaqMan(AbD)	cSNP	RAB6A intron	
	rs1048234	TaqMan(AbD)	cSNP	RAB6A 5'UTR	
11q13.4	rs161054	TaqMan(AbD)	cSNP	MRPL48 intron	Not polymorphic
	rs1782174	TaqMan(AbD)	cSNP	MRPL48 5'UTR	
	rs1728534	TaqMan(AbD)	cSNP	MRPL48 intron	Only heterozygote
	rs1782180	TaqMan(AbD)	cSNP	MRPL48 intron	
	rs1453825	TaqMan(AbD)	cSNP	MRPL48 intron	Not polymorphic
	rs1792193	TaqMan(AbD)	cSNP	MRPL48 intron	

Continued

Cyto- bands	SNPs	Method	g-SNP	Location gene name portion	Note
11q13.4	rs1051080	TaqMan(AbD)	cSNP	MRPL48 5'UTR	
	rs2010583	TaqMan(AbD)	cSNP	MRPL48 5'UTR	
	rs1870581	TaqMan(AbD)	cSNP	FLJ11848 intron	Not polymorphic
	rs2067912	TaqMan(AbD)	cSNP	FLJ11848 intron	
	rs3741138	TaqMan(AbD)	cSNP	FLJ11848 intron	
	rs1818529	TaqMan(AbD)	cSNP	FLJ11848 intron	Not polymorphic
	rs36365	TaqMan(AbD)	cSNP	FLJ11848 intron	
	rs373228	TaqMan(AbD)	cSNP	LOC374407 syn	
	rs533263	TaqMan(AbD)	cSNP	LOC374407 syn	
	rs655717	TaqMan(AbD)	cSNP	UCP2 intron	
10p13	rs660339	TaqMan(AbD)	cSNP	UCP2 intron	
	rs660514	TaqMan(AbD)	cSNP	UCP3 syn	
	rs2073577	TaqMan(AbD)	cSNP	UCP3 intron	Not polymorphic
	rs2229706	TaqMan(AbD)	cSNP	UCP3 intron	
	rs1800849	TaqMan(AbD)	cSNP	UCP3 intron	
	rs1320428	TaqMan(AbD)	cSNP	UCP3 intron	Not polymorphic
	rs1885343	TaqMan(AbD)	cSNP	UCP3 intron	
	rs620072	TaqMan(AbD)	cSNP	DKFZP588P0123 intron	
	rs620032	TaqMan(AbD)	cSNP	DKFZP588P0124 intron	Not polymorphic
	rs660503	TaqMan(AbD)	cSNP	DKFZP588P0123 intron	
10p13	rs1327302	TaqMan(AbD)	cSNP	DKFZP588P0123 intron	
	rs2493762	TaqMan(AbD)	cSNP	DKFZP781F241 3'UTR	Not polymorphic
	rs963335	TaqMan(AbD)	cSNP	DKFZP781F241 intron	
	rs2280076	TaqMan(AbD)	cSNP	DKFZP781F241 intron	
	rs1439915	TaqMan(AbD)	cSNP	DKFZP781F241 intron	
	rs660762	TaqMan(AbD)	cSNP	DKFZP781F241 intron	
	rs660822	TaqMan(AbD)	cSNP	DKFZP781F241 intron	Not polymorphic
	rs2958207	TaqMan(AbD)	cSNP	DKFZP781F241 intron	
	rs662141	TaqMan(AbD)	cSNP	DKFZP781F241 intron	
	rs620408	TaqMan(AbD)	cSNP	DKFZP781F241 intron	
14q23.1	rs3957895	TaqMan(AbD)	cSNP	OPTN intron	Not polymorphic
	rs660580	TaqMan(AbD)	cSNP	OPTN intron	
	rs1347979	TaqMan(AbD)	cSNP	OPTN intron	
	rs71068	TaqMan(AbD)	cSNP	OPTN intron	
	rs2590915	TaqMan(AbD)	cSNP	OPTN intron	Not polymorphic
	rs660582	TaqMan(AbD)	cSNP	OPTN intron	
	rs2244380	TaqMan(AbD)	cSNP	OPTN intron	
	rs765884	TaqMan(AbD)	cSNP	OPTN intron	
	rs1822343	TaqMan(AbD)	cSNP	OPTN intron	Not polymorphic
	OPTN-1	TaqMan(AbD)	cSNP	OPTN intron	
14q23.1	rs1324252	TaqMan(AbD)	cSNP	OPTN intron	
	rs725951	TaqMan(AbD)	cSNP	OPTN intron	
	rs2073318	TaqMan(AbD)	cSNP	RTN1 intron	
	rs1800579	TaqMan(AbD)	cSNP	RTN1 intron	
	rs1805789	TaqMan(AbD)	cSNP	RTN1 intron	
	rs184737	TaqMan(AbD)	cSNP	RTN1 intron	
	rs2348698	TaqMan(AbD)	cSNP	RTN1 intron	
	rs1802043	TaqMan(AbD)	cSNP	RTN1 intron	
	rs1857989	TaqMan(AbD)	cSNP	RTN1 intron	
	rs2182138	TaqMan(AbD)	cSNP	RTN1 intron	
14q23.1	rs1852041	TaqMan(AbD)	cSNP	RTN1 intron	Multi cluster
	rs1857996	TaqMan(AbD)	cSNP	RTN1 intron	Not polymorphic
	rs1852032	TaqMan(AbD)	cSNP	RTN1 intron	
	rs1857983	TaqMan(AbD)	cSNP	RTN1 intron	Multi cluster
	rs253268	TaqMan(AbD)	cSNP	RTN1 intron	
	rs273226	TaqMan(AbD)	cSNP	RTN1 intron	
	rs2084862	TaqMan(AbD)	cSNP	RTN1 intron	
	rs1851353	TaqMan(AbD)	cSNP	RTN1 intron	Multi cluster

*1: Typed by TaqMan systems. Primers and probes were prepared by Assays-on-Demand™

*2: Typed by direct sequencing

Used primers

SNPs on BSL (6p21.3)

Template PCR forward 5'-GCAAGAGATGAGGCGCTAACCTAAC-3'

Template PCR reverse 5'-CATCTACGATAGTCTTCTCGGTC-3'

Sequencing primer 5'-TACCTGCGCTGCTGAGGCT-3'

Sequencing primer 5'-AGAGCTGGAGAGCGGAG-3'

SNPs (rs20692-rs2071283, rs2071281, rs15929) on NOTCH4 (6p21.3)

Template PCR forward 5'-TCCTTCTCTACCTCCCACTCTCTGA-3'

Template PCR reverse 5'-CACTCTGCTGCGGCGGATACGAC-3'

Sequencing primer 5'-GGCTGAGGTGAGGAGTGCCAG-3'

SNPs (rs367888) on NOTCH4 (6p21.3)

Template PCR forward 5'-GGCTGAGGTCTTCATGTGCGGATC-3'

Template PCR reverse 5'-GGTGTCCAGGACATTTGTGACACA-3'

Sequencing was performed using reverse primer.

SNPs on RAB6A (11q13.4)

Template PCR forward 5'-CAGGCAAGCATGATGATTG-3'

Template PCR reverse 5'-TCCATTGAGGAGCTATATGG-3'

Sequencing was performed using reverse primer.

*3: Typed by TaqMan systems. Primers and probes were prepared by Assays-on-Demand™

Samples for pooled screens

22 *Nonnenmannia* S.M. P.

3 Fisher's exact test P -value is .02 table of values

[Table 8]

Table 3. LD blocks and haplotype association with RA													
Cytobands name size (kb)	Block* SNPs		Included genes name	Number of SNPs	Number of haplotype	Positive haplotype	Haplotype frequencies		Fisher's exact P values		Odds Ratio	95%CI	
	end	start					Control	Case	95%CI	2x2			
6p21.3	a1	8.26	rs2071595 - rs2071592	5	5	4	0.074	0.055-0.093	0.028	0.019-0.040	0.000033	0.35	0.21-0.59
a2	9.03	rs2239708 - rs2071591	IBKL	2	3	4	0.051	0.045-0.485	0.485	0.459-0.531	1	1.51	0.97-1.46
a3	19.03	rs2269475 - rs1046809	IBKL	3	4	1	0.008	0.003-0.015	0.012	0.005-0.020	0.10	1.51	0.53-4.26
a4	127.97	rs2242656 - rs707929	BAT3-C6orf27	10	11	4	0.072	0.056-0.090	0.030	0.019-0.041	0.00014	0.39	0.23-0.64
a5	4.13	rs2242668 - rs2075800	LSM2-HSPAIL	3	3	2	0.126	0.102-0.150	0.089	0.070-0.109	0.024	0.88	0.49-0.94
a6	58.45	rs2242569 - rs429150	TVNG	9	9	2	0.362	0.328-0.399	0.084	0.073-0.286	0.000048	0.83	0.50-0.78
a7	9.75	rs206018 - rs2649012	NOTCH4	2	3	1	0.073	0.040-0.706	0.774	0.745-0.805	0.000015	1.66	1.32-2.09
a8	0.85	rs422951 - rs415929	NOTCH4	8	3	1	0.103	0.083-0.126	0.203	0.175-0.231	0.0000010	2.20	1.64-2.95
a9	0.02	rs915894 - rs443198	NOTCH4	2	2	2	0.493	0.460-0.531	0.472	0.400-0.465	0.020	0.78	0.64-0.96
DRB1	0.26	rs2308754 - rs1141742	DRB1	64	29	*0405	0.129	0.089-0.276	0.326	0.014-0.037	0.00000000000014	2.67	2.04-3.49
a10	16.35	rs2071788 - rs2071550	DQB2	3	4	4	0.069	0.052-0.089	0.025	0.014-0.037	0.000077	0.35	0.20-0.60
11q13.4	b1	6.86	rs2008734 - rs6590	2	3	2	0.412	0.376-0.447	0.479	0.445-0.513	0.011	1.31	1.07-1.61
b2	139.37	rs1792117 - rs935985	MRPL48 - FLJ11848	8	6	1	0.500	0.481-0.533	0.595	0.565-0.629	0.021	1.48	1.21-1.82
b3	4.99	rs655717 - rs660339	UCP2	2	2	2	0.454	0.417-0.489	0.535	0.501-0.569	0.00027	1.38	1.12-1.69
b4	9.02	rs668514 - rs2075577	UCP3	2	3	2	0.352	0.320-0.387	0.273	0.240-0.308	0.0010	0.89	0.55-0.86
b5	17.81	rs668650 - rs1527302	DKFZP568P0123	2	3	2	0.353	0.320-0.388	0.267	0.238-0.297	0.00044	0.87	0.54-0.84
10p13	c1	3.89	rs1347979 - rs571086	2	4	3	0.176	0.150-0.203	0.120	0.097-0.145	0.00035	0.84	0.49-0.86
c2	6.07	rs2244380 - rs765884	OPTN	2	3	3	0.101	0.079-0.123	0.121	0.100-0.145	0.25	1.22	0.89-1.69
c3	13.64	rs996999 - rs1324252	OPTN	2	3	3	0.039	0.027-0.052	0.047	0.032-0.083	0.52	1.22	0.74-2.01
14q23.1	d1	28.86	rs1952043 - rs2182138	3	5	3	0.207	0.180-0.238	0.158	0.133-0.185	0.014	0.72	0.65-0.83
d2	19.22	rs927326 - rs2064992	RTN1	2	4	2	0.457	0.423-0.493	0.509	0.472-0.545	0.050	1.23	1.01-1.51

LD blocks were inferred by the EM algorithm.

Expression Analysis

To study the expression patterns of these genes in various tissues including synovial cells, we performed quantitative reverse transcription-PCR (QRT-PCR) using RNA from these tissues.

Total RNA was isolated by ISOGEN (Nippon Gene) from synovial membranes surgically obtained from eight RA and four osteoarthritis (OA) patients. We also isolated total RNA from a synovial cell line (SW982) provided by American Type Culture Collection (ATCC). Other RNAs from various tissues are commercially available from Clontech, Invitrogen, Origene, and Stratagene. We evaluated the quality and quantity of these RNAs by use of Agilent 2100 Bioanalyzer (Agilent) and confirmed their quantities by RiboGreen RNA fluorescence assay (Molecular Probes). Complimentary DNAs were synthesized from these total RNAs using random hexamers and TaqMan reverse transcription reagents kit (Applied Biosystems). We obtained cDNA-specific primers and probes by the 'Assay-by-Design (AbD)' for the ten genes tested and by the 'Assay-on-Demand (AoD)' for GAPD used as a housekeeping control gene, all of which were provided by Applied Biosystems. After preliminary experiments, 210 nM probes, 756 nM primers, and 0.48 ng/ml cDNA at the final concentration in 50 µl reaction volume were used in 96-well reaction plates on ABI PRISM 7900 according to the standard approach recommended by Applied Biosystems. Each plate was processed three times to calculate the average and SD for each sample. Estimated quantity was calculated

each time using a standard curve in each well. All quantity data normalized to GAPD were tested by the Smirnov's test with a 5% significance level. After the reciprocal transformation of all the normalized quantity data, the Student's t-test was conducted for expression levels between RA and OA synovial tissues.

The consistently high expression of NOTCH4 in the lung and of TNXB in the adrenal gland were observed (Figure 3a). Our results also showed that all the genes were expressed in the RA synovial cells. TNXB and NOTCH4 had significantly high expression levels in the RA synovial cells, whereas RTN1 had the lowest level. We also compared the expression levels of these genes between RA and OA synovial cells. The expression levels of the MRPL48 ($P=0.049$) and DKFZP761F241 ($P=0.027$) genes exhibited relatively significant difference between the RA and OA synovial cells by the Student's t-test (Table 9 and Figure 3b). MRPL48 expression in the RA synovial tissue was approximately twice that in the OA tissue. Three-quarters of the RA tissue donors were homozygous for a positive haplotype in the block b2 of the MRPL48 locus.

[Table 9]

Table 4. Expression levels of RA candidate genes in OA and RA synovial cells

Gene	OA synovial cell		RA synovial cell	
	Average	S.D.	Average	S.D.
IkBL	3.1	2.4	1.6	0.8
TNXB	339.3	349.7	80.7	35.1
NOTCH4	36.7	0.4	39.7	30.8
MRPL48	2.5*	0.2	4.4	1.6
FLJ11848	0.9	0	1.3	0.7
UCP2	1.5	0.9	2.8	2.1
DKFZP761F241	17.5*	8.1	7.1	0.8
OPTN	7.4	2.4	12.7	3.4
RTN1	0.8	0.1	0.9	0.6
BLT2**	51.2	70.7	11.1	6.9

* Expression levels of MRPL48 (P=0.049) and DKFZP761F241 (P=0.027) genes showed relatively significant difference between RA and OA synovial tissues.

** The BLT2 (leukotriene B4 receptor subtype 2) gene was employed as a positive control, which has been known to have strong expression in the RA

Statistical Analysis:

To calculate P-values, two types of the Fisher's exact test were used for the 2x2 contingency tables for each individual allele and the 2xm contingency tables for each locus. In this context, m refers to the number of markers observed in a population. To practice the Fisher's exact test for the 2xm contingency tables, Markov chain/Monte Carlo simulation method was adopted. We simply presented "allelic" but not

phenotypic association for the 2x2 contingency tables for MS, SNP and haplotype. These P-values were corrected by Bonferroni's correction, wherein the coefficient was the total number of the contingency tables tested. These analyses were conducted with software package MCFishman. Other basic statistical analyses including multiple logistic regression analysis and Mantel-Haenszel test were performed using SPSS program package and Microsoft Excel (trade name). We predicted LD block structures for these SNPs by using the confidence intervals of the D' value as a LD measure (Gabriel, S. B. et al., Science, 296, 2225 (2002); and Dawson, E. et al., Nature, 418, 544 (2002)). Moreover, haplotypes in each block and their frequencies were estimated by EM and Clark algorithms. Finally, to evaluate the reliability of haplotypes in each block, the 95% confidence interval was calculated from each haplotype frequency given by bootstrap resampling of up to 2000 times on the basis of the estimated haplotype frequencies, which was implemented in the Right program (Mano. S. et al., Ann. Hum. Genet., in press).

In this Example, strong association was found in TNXB and NOCTH4 genes 250 kb distant from HLA-DRB1 in the candidate region narrowed down by the MS markers. These genes are known to be located in LD blocks evidently different from that of HLA-DRB1 (Cullen, M. et al., Am. J. Hum. Genet., 71, 759 (2002); and Walsh, E. C. et al., Am. J. Hum. Genet., 73, 580 (2003)). In agreement with the multiple logistic regression analysis result, the result of Mantel-Haenszel test also showed that

positive SNPs in TNXB and NOTCH4 are independent of HLA-DRB1*0405 or SE in both partially dominant and partially recessive models (data not shown). Further, the candidate region was highly identical to one of additional susceptibility regions previously predicted (Jawaheer, D. et al., Am. J. Hum. Genet., 71, 585 (2002)). TNXB is known as a causative gene of one type of Ehlers-Danlos syndrome (MIM*600985) characterized by dysfunction in connective tissues including joints. Its gene products participate in connective tissue functions and in structures via the deposition of collagens of various types (Mao, J. R. et al., Nat. Genet., 30, 421 (2002)), probably including synovial tissues shown here. Type II collagen-induced arthritis in mice is known to mimic rheumatoid arthritis (Moore, A. R., Methods Mol. Biol., 225, 175 (2003)).

The present inventors believe that the amino acid exchanges of the TNXB gene product serve as functional factors for RA via a hypothetical pathway associated with collagen metabolism. In recent years, it was reported that the NOTCH4 gene product might participate in overproliferation via tumor necrosis factor (TNF) of synovial cells and in RA (Ando, K. et al., Oncogene, 22, 7796 (2003)). However, large parts of NOTCH4 function are still unclear.

On 11q13.4, although MRPL48 function is still unknown, its expression pattern indicated the association of this gene with RA. The candidate region 11q13.4 contains other interesting genes RAB6A, FLJ11848, UCP2, and UCP3. As with this region, even though further association analysis for 10q13

and 14q23.1 requires using higher-density SNP markers, it is interesting that other chromosomes were found by the method of the present invention. These results chiefly suggested that our marker set and method are highly practicable and applicable to other complicated diseases, at least to oligogene diseases with major genes such as HLA-DRB1 in RA.

Interestingly, our data suggested that the seven most significant MS markers are individually positioned in particular LD blocks as a trend (Figure 3). These markers were observed on the "Clark blocks" rather than the "EM blocks". In many cases, positive MS alleles were obviously associated with positive SNP haplotypes in these blocks.